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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07D 207/46, 403/12

A1

(11) International Publication Number: WO 90/13540

(43) International Publication Date: 15 November 1990 (15.11.90)

(21) International Application Number: PCT/US90/02133

(22) International Filing Date: 19 April 1990 (19.04.90)

(30) Priority data:
340,928 19 April 1989 (19.04.89) US

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(81) Designated States: AT (European patent), AU, BE (European patent), CA, CH (European patent), DE (European patent), DK (European patent), ES (European patent), FR (European patent), GB (European patent), HU, IT (European patent), JP, LU (European patent), NL (European patent), SE (European patent), SU.

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: ACTIVE CARBONATES OF POLYALKYLENE OXIDES FOR MODIFICATION OF POLYPEPTIDES

(57) Abstract

Poly(ethylene glycol)-N-succinimide carbonate and its preparation are disclosed. Polyethylene glycol (PEG) is converted into its N-succinimide carbonate derivative. This form of the polymer reacts readily with amino groups of proteins in aqueous buffers. The modified proteins have PEG-chains grafted onto the polypeptide backbone by means of stable, hydrolysis-resistant urethane (carbamate) linkages.

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ACTIVE CARBONATES OF POLYALKYLENE OXIDES FOR MODIFICATION OF POLYPEPTIDES

This is a continuation-in-part of U.S. Serial No. 340,928, filed April 19, 1989, the contents of which are hereby incorporated by reference into the present application.

BACKGROUND OF THE INVENTION

The present invention relates to chemical modification of polypeptides by means of covalent attachment of strands of polyalkylene oxide to a polypeptide molecule such as is disclosed in U.S. Patent No. 4,179,337, to Davis, et al. It is disclosed in Abuchowski & Davis "Enzymes as Drugs", Holcenberg & Roberts, eds., pp. 367-383, John Wiley & Sons, N.Y. (1981) that such preparations of polypeptides have reduced immunogenicity and antigenicity and also have a longer lifetime in the bloodstream as compared to the parent polypeptides. These beneficial properties of the modified polypeptides make them very useful in a variety of therapeutic applications, such as enzyme therapy.

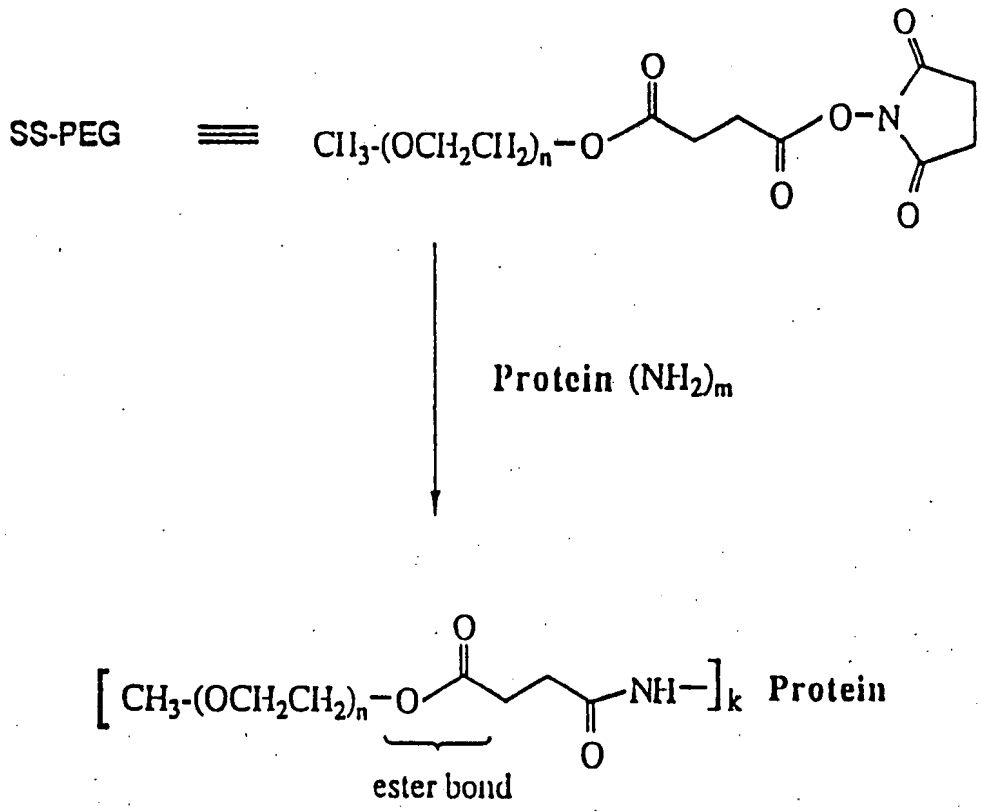
The active groups that are introduced onto polyalkylene oxides for the purpose of subsequent attachment of these polymers to proteins must satisfy the following requirements:

1. The active groups have to be reactive enough to afford fast reaction with a protein under mild conditions;

2. The residues released from the active groups during the process of modification have to be non-toxic and/or readily separable from the protein-polymer adduct.

To effect covalent attachment of polyethylene glycol (PEG) to a protein, the hydroxyl end-groups of the polymer must first be converted into reactive functional groups. This process is frequently referred to as "activation" and the product is called "activated PEG". Methoxypolyethylene glycol (mPEG) derivatives, capped on one end with a functional group, reactive towards amines on a protein molecule, are used in most cases.

The most common form of activated PEG heretofore used for preparation of therapeutic enzymes is poly(ethylene glycol) succinoyl-N-hydroxysuccinimide ester (SS-PEG) [Abuchowski, et al. Cancer Biochem. Biophys. 7, 175-186 (1984), Scheme 1]. Use of this activated polymer satisfies both of the requirements listed above. However, it has one major drawback. The ester linkage between the polymer and succinic acid residue has limited stability in aqueous media [U.S. Patent No. 4,670,417, to Iwasaki, et al. (1987); Ulbrich, et al., Makromol. Chem. 187, 1131-1144 (1986)]



Scheme 1: Conventional attachment of PEG to a protein using SS-PEG as the activated form of the polymer

Various functionalized polyethylene glycols (PEG) have been effectively used in such fields as protein modification (Abuchowski & Davis, 1981, supra), peptide chemistry [Zalipsky, et al., Int. J. Peptide Protein Res., 30, 740-783 (1987)] and preparation of conjugates with biologically active materials [Zalipsky, et al., Eur. Polym. J. 19, 1177-1183 (1983) and Zalipsky and Barany, Polymer Preprints, Am. Chem. Soc. Div. Polym. Chem. 27(1), 1-2 (1986)]. PEG protein conjugates useful in medical applications have shown promise, particularly with regard to their stability to

proteolytic digestion, reduced immunological response and longer half-life times in the bloodstream.

To accomplish this, the prior art has activated the hydroxy group of PEG with cyanuric chloride and the resulting compound then coupled with proteins (Abuchowski, et al. (1977) J. Biol. Chem. 252, 3578; Abuchowski and Davis, 1981, supra). However, various disadvantages of using this method exist, such as the toxicity of cyanuric chloride and the non-specific reactivity for proteins having functional groups other than amines, such as free essential cysteine or tyrosine residues.

In order to overcome these and other disadvantages, alternative procedures, such as succinimidyl succinate derivatives of PEG (SS-PEG) have been introduced (Abuchowski, et al. 1984, supra, see Scheme 1, above). It reacts quickly with proteins (30 min) under mild conditions yielding active yet extensively modified conjugates use of this activated polymer has one major disadvantage. The ester linkage between the polymer and the succinic acid residue has limited stability in aqueous media [U.S. Patent No. 4,670,417 to Iwasaki, et al. and Ulbrich, et al. Makromol Chem., 187, 1131-1144 (1986)].

Formation of urethane linkages between amino groups of a protein and PEG overcomes the problem of hydrolytic loss of the polymer chains [Veronese, et al., Appl. Biochem. Biotechnol. 11, 141-152 (1985)]. In fact, it was demonstrated on radioactively labeled PEG-derivatives that urethane links are completely stable under a variety of physiological conditions [Larwood & Szoka J., Labeled Compounds Radiopharm., 21, 603-614 (1984)]. The attachment of PEG to a protein via a carbamate derivative was accomplished [Beauchamp, et al. Analyt. Biochem. 131, 25-33

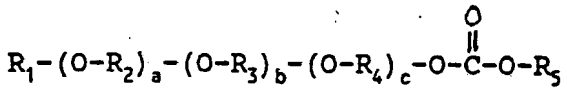
(1983)] using carbonyldiimidazole-activated PEG. However, the polymer activated in this manner is not very reactive and therefore long reaction times (48 - 72 hrs at pH 8.5) were required to achieve sufficient modifications. Therefore, the carbonyldiimidazole-activated agent clearly does not satisfy the first requirement noted above. An additional disadvantage of this approach is in the relatively high cost of carbonyldiimidazole.

Use of PEG-phenylcarbonate derivatives for preparation of urethane-linked PEG-proteins was reported [see Veronese, et al. (1985), supra]. The main drawback of this approach lies in the toxicity of the hydrophobic phenol residues (p-nitrophenol or 2, 4, 5-trichlorophenol) and their affinity for proteins. Clearly this method does not satisfy the second requirement noted above.

Each of the activated forms of the polymer has properties which can be considered advantageous or disadvantageous, depending on the system of use. In light of the many applications of PEG-polypeptides, it is desirable to broaden the of protein modifying PEG-reagents made for a specific end use.

SUMMARY OF THE INVENTION

The present invention provides a compound having the structure



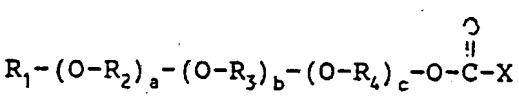
wherein R_1 is H-, H_3C-, an N-dicarboximide group, or any other functional group;

wherein each R_2, R_3, and R_4 is an alkyl group which may be straight, branched, disubstituted, or unsubstituted, and wherein each R_2, R_3, and R_4 may be independently the same as, or different from, the others of R_2, R_3, and R_4;

wherein R_5 is an N-dicarboximide group; and

wherein a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b, and c is between 10 and 1000.

The present invention also provides a process for preparing the compound which comprises reacting a compound having the structure



wherein R_1 is H-, H_3C-, N-dicarboximide group, or any other functional group;

wherein each R_2, R_3, and R_4 is an alkyl group which may be straight, branched, disubstituted, or unsubstituted, and wherein each R_2, R_3, and R_4 may be independently the same as, or different from, the others of R_2, R_3, and R_4;

wherein X is a halogen;

wherein a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b, and c is between 10 and 1000.

with an N-hydroxydicarboximide in the presence of a base.

The present invention further provides a modified polypeptide comprising a polypeptide having bound thereto at least one polymer having the structure

$$R_1-(O-R_2)_a-(O-R_3)_b-(O-R_4)_c-O-\overset{O}{\overset{\|}{C}}-$$

wherein R_1 is H-, H_3C-, or a succinimide carbonate group;

wherein each R_2, R_3, and R_4 is an alkyl group which may be straight, branched, substituted, or unsubstituted, and wherein each R_2, R_3, and R_4 may be independently the same as, or different from, the others of R_2, R_3, and R_4;

wherein a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum

of a, b, and c is between 10 and 1000.

wherein each polymer is covalently bound to an amine group of the polypeptide by a urethane linkage.

The invention also provides a process for preparing a modified polypeptide comprising reacting a polypeptide with the compound at a pH in the range of about 5.8 - 11.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. Release of mPEG from PEG-BSA conjugates.

Experimental conditions: Solutions of both types of PEG-BSA conjugates (~61% modification) at concentration 4 mg/ml (by Bluret assay) were incubated in the appropriate buffer. At given time intervals aliquots of these solutions were injected into an HPLC equipped with Zorbax GF-450 column and RI-detector to quantitate mPEG-5000.

Figure 2. Stability of SS-PEG and SC-PEG at 22°C.

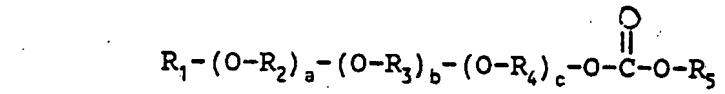
Experimental conditions: The activated PEG's were stored in the form of fine powder in tightly closed polypropylene containers. At given time intervals samples of each polymer were assayed for active acyl content.

Figure 3. Reactivity of activated PEG's as a function of pH.

Experimental conditions: To triethanolamine-borate buffer (0.3 M, 1 ml) at the appropriate pH, stock solution of NAL in water (50 mM, 0.1 ml) was added followed by stock solution of the appropriate activated PEG in CH_3CN (50 mM active acyl, 0.1 ml). The resultant solution was vortexed and incubated at 28°C for 1 hr. A mixture of the same components but leaving out SX-PEG was used as a control. The TNBS - assay version of Snyder, et al. [(1975) Anal. Biochem. 64, 284] was used to determine the unreacted NAL.

DESCRIPTION OF THE INVENTION

The present invention describes activated polyalkylene oxides having the general structure:



wherein R_1 is H-, H_3C-, an N-dicarboximide group, or any other functional group; wherein each R_2, R_3, and R_4 is an alkyl group which may be straight, branched, disubstituted, or unsubstituted, and wherein each R_2, R_3, and R_4 may be independently the same as, or different from, the others of R_2, R_3, and R_4; wherein R_5 is an N-dicarboximide group; and wherein a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b, and c is between 10 and 1000.

More specifically, the invention relates to preparation and use of a new, activated PEG, namely, poly(ethylene glycol)-succinimide carbonate (SC-PEG) and the bifunctional derivative of PEG, namely, polyethylene glycol-bis-succinimide carbonate (BSC-PEG). Furthermore, heterobifunctional derivatives of PEG are possible (Zalipsky and Barany, supra); one of the end groups is succinimide carbonate and the other end group (R^1, see Scheme 2, infra) contains a different reactive functional group, such as a free carboxyl group or an amino acid. These materials react readily with amino groups of proteins to afford PEG attachment through stable urethane linkages (Scheme 2, below). The reactivity of the new agents, SC-PEG and BSC-PEG, are comparable to the conventionally used SS-PEG. Thus, high degrees of modification are achievable in mild conditions (aqueous buffers, pH 5.8-11, preferably pH 7.0-

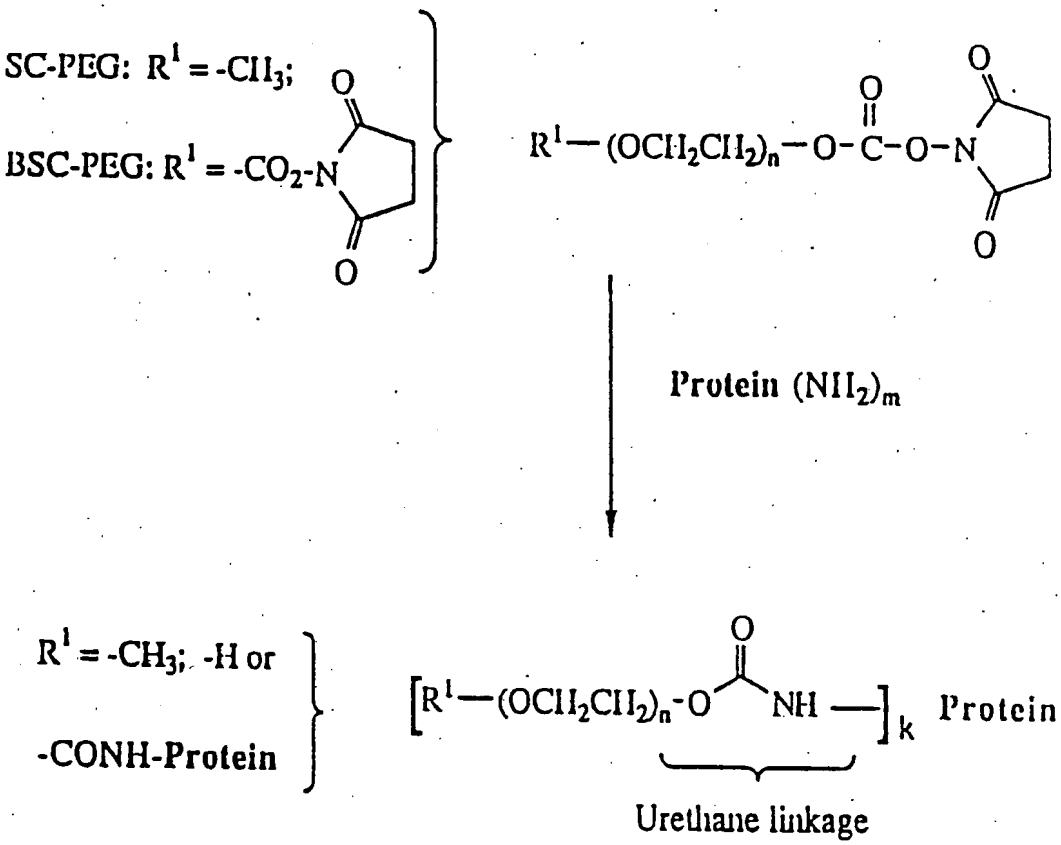
9.5) within about 30-60 min. and moderate temperatures (4-40°C). Additionally, the agents are soluble in a variety of organic solvents, thus being useful and important in the coupling of low molecular weight, partially protected peptides and other biologically useful ligands.

The PEG does not have to be of a particular molecular weight, but it is preferred that the molecular weight be between 500 and 40,000; more preferably between 2,000 and 20,000. The choice of molecular weight of PEG is made based on the nature of the particular protein employed, for example, the number of amino groups available for modification.

The N-hydroxysuccinimide released during protein modification is non-toxic material that is often used in protein chemistry, especially for preparation of biologically active protein-adducts. As in the case of above mentioned carbonyldiimidazole activated PEG and PEG-phenylcarbonates, the product of protein modification using SC-PEG or BSC-PEG has PEG-chains grafted onto the polypeptide backbone through carbamate (urethane) linkages. However, due to the higher reactivity of the new agents, higher degrees of modification are achievable in shorter periods of time. An additional advantage of succinimide carbonate activated PEG is that those active functional groups that do not react with amino groups of a protein undergo fast aqueous hydrolysis producing N-hydroxysuccinimide, carbon dioxide and hydroxy-terminated PEG. This is of particular importance in the case of bifunctional PEG derivatives (BSC-PEG). These materials can serve a dual purpose: PEGylation and crosslinking at the same time. The BSC-PEG, like any homobifunctional material can be used to crosslink two different proteins. When a BSC-PEG molecule reacts with a protein via only one end

group, the other SC-group of the polymer, which does not react with the amine, is hydrolyzed and therefore no extraneous (potentially antigenic) residues are introduced onto the PEG-protein conjugate.

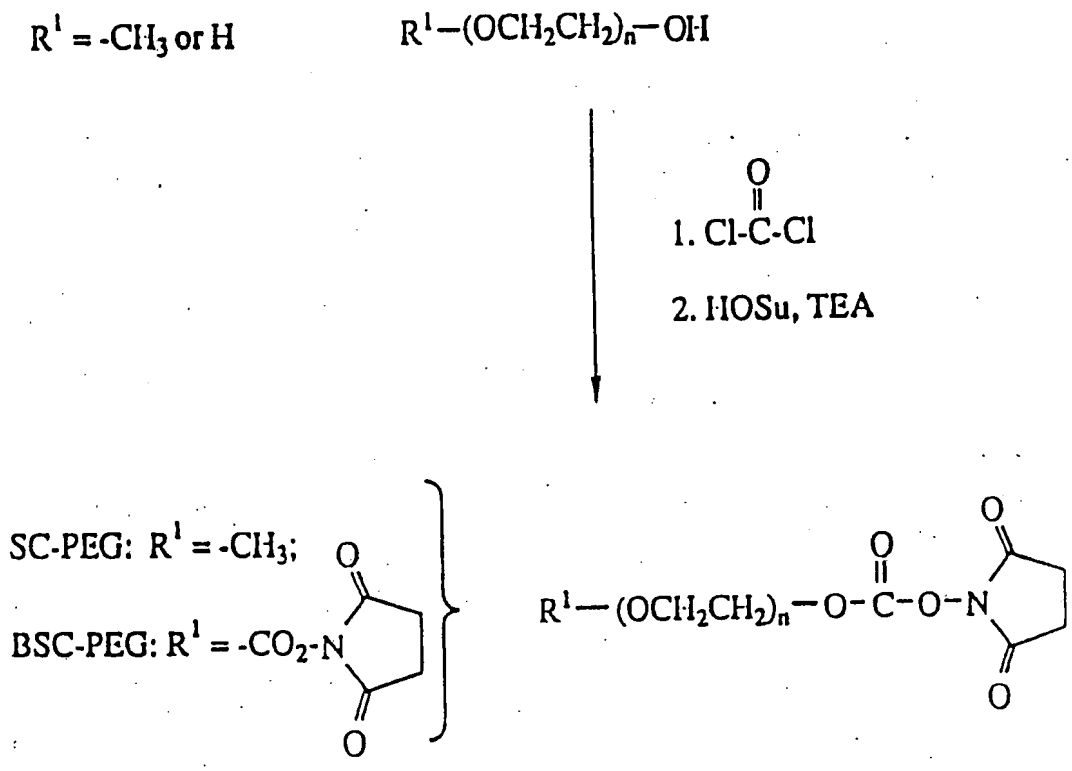
Biological activities of proteins modified with SC-PEG and BSC-PEG are preserved to a large extent as shown by the examples below. There is one literature precedent in which protein (tissue plasminogen activator) covalently conjugated with PEG via urethane links had a higher specific activity than the same protein modified with SS-PEG to approximately the same extent [Berger & Pizzo, Blood, 71, 1641-1647



Scheme 2: Use of SC-PEG and BSC-PEG for attachment of PEG to proteins.

Naturally, the utility of SC-activated PEG-derivatives extends to preparation of PEG-conjugates of low molecular weight peptides and other materials that contain free amino groups.

A one-pot procedure for introduction of SC-groups onto PEG was developed (Scheme 3 below). First, polyethylene glycol chloroformate was generated *in situ* by treatment of the polymer (PEG) with phosgene. The resulting chloroformate was then reacted with N-hydroxysuccinimide (HOSu) followed by triethylamine (TEA) to yield the desired activated derivatives of PEG. The activated polymer preparations were purified from low molecular weight materials and determined to contain the theoretical amounts of active groups.



Scheme 3: Synthesis of N-succinimide carbonate derivatives of poly(ethylene glycol).

Example 1

Preparation of SC-PEG: Methoxypolyethylene glycol of molecular weight 5000 (Union Carbide, 60 g, 12 mmol) was dissolved in toluene/dichloromethane (3:1, 200 ml) and treated with a toluene solution of phosgene (30 ml, 57 mmol) overnight. The solution was evaporated to dryness and the remainder of phosgene was removed under vacuum. The residur was redissolved in toluene/dechloromethane (2:1, 150 ml) and treated with solid N-hydroxysuccinimide (2.1 g, 18 mmol) followed by triethylamine (1.7 ml, 12 mmol). After 3 hours, the solution was filtered and evaporated to dryness. The residue was dissolved in warm (50°C) ethyl acetate (600 ml), filtered from trace insolubles and cooled to facilitate precipitation of the polymer. The product was collected by filtration and then recrystallized once more from ethylacetate. The product was dried in vacuo over P_2O_5. The yield was 52.5 g (85% of theory).

To determine the active carbonate content of the product, samples of the polymer were reacted with a measured amount of benzylamine in dichloromethane and the excess of amine was titrated with perchloric acid in dioxane. These titrations indicated that 1 g of the product contained 1.97 x 10^{-4} mole of active carbonate (101% of theoretical content). I.R. (film on NaCl, cm^{-1}) characteristic bands at : 1812 and 1789 (both C=O, succinimide); 1742 (C=O, carbonate); 1114 (CH_2OCH_2). ^{13}C-NMR ($CDCl_3$):δ 168.5 ($CH_2\underline{C}$=O); 151.3 (O-CO_2); 71.9 ($CH_3O\underline{C}H_2$) 70.2 (PEG); 68.7 ($\underline{C}H_2CH_2OCO_2$); 68.0 ($CH_2\underline{C}H_2OCO_2$); 58.9 ($CH_3O$); 25.2 ($\underline{C}H_2C$=O) ppm.

Example 2

Preparation of BSC-PEG: Polyethylene glycol of molecular

weight 4600 (Union Carbide, 50g, 21.7 mequiv. OH) was converted to the corresponding bis-N-hydroxysuccinimide carbonate using a toluene solution of phosgene (50 ml, 96.5 mmol) and then N-hydroxysuccinimide (3.8 g, 23 mmol) and triethylamine (3.2 ml, 23 mmol) following the procedure described in Example 1. After purification the product was obtained as a white powder (51 g, 96%). Active carbonate content was 4.0×10^{-4} mole/g (98% of theoretical) as determined by titrations with benzylamine-perchloric acid. I.R. (film on NaCl, cm^{-1}) characteristic bands at : 1812 and 1789 (both C=O, succinimide); 1742 (C=O, carbonate); 1114 (CH_2OCH_2). ^{13}C-NMR($CDCl_3$): δ 168.5 ($CH_2\underline{C}$=O); 151.3 (O-CO_2); 70.2 (PEG); 68.7 ($\underline{C}H_2CH_2OCO_2$); 68.0 ($CH_2\underline{C}H_2OCO_2$); 25.2 ($\underline{C}H_2C$=O) ppm. H-NMR ($CDCl_3$):$\delta$ 4.35 (m, 4H, CH_2OCO_2); 3.55 (s, ~ 400H, PEG); 2.74 (s, 8H, CH_2C=O) ppm.

Example 3

Preparation of polyethylene glycol-Bovine Serum Albumin conjugates (PEG-BSA):

A. SC-PEG (1 g) was added to a stirred solution of Bovine Serum Albumin (BSA) (100 mg) in 0.1 M sodium phosphate, pH 7.8 (20 ml). Sodium hydroxide (0.5 N) was used to maintain pH 7.8 for 30 min. The excess of free PEG was removed by diafiltration using 50 mM phosphate buffered saline. The extent of modification of BSA was approximately 50% (30) as determined by trinitrobenzenesulfonate (TNBS) titration of amino groups [Habeeb, Analyt. Biochem. 14, 328-336 (1966)].

The same degree of modification was obtained when the experiment was repeated under identical conditions using SS-PEG instead of SC-PEG.

B. SC-PEG (1 g) was added to a stirred solution of BSA (100 mg) in 0.1 M sodium borate, pH 9.2. Sodium hydroxide (0.5 N) was used to maintain pH 9.2 for 30 min. The excess of free PEG was removed by diafiltration and the product assayed for the number of free amino groups. Approximately 68% (41) of the amino groups of the native BSA were modified.

C. BSC-PEG (1 g) was added to a stirred solution of BSA (100 mg) in 0.1 M sodium borate, pH 9.2. Sodium hydroxide (0.5 N) was used to maintain pH 9.2 for 30 min. The excess of free PEG was removed by diafiltration and the product assayed for the number of free amino groups. Approximately 80% (48) of the amino groups of the native BSA were modified. Analysis of the product by HPLC (Gel Filtration) indicated that over 65% of PEG-BSA was in intermolecularly crosslinked form and about 35% of the product had the same molecular weight as PEG-BSA from Example 3B.

Example 4

Preparation of PEG-glutaminase: A solution of glutaminase Pseudomonas 7A (200 mg) in 0.1 M sodium phosphate, pH 7.8 was treated with SC-PEG (4.7) g). The solution was stirred and pH 7.8 was maintained for 30 minutes. The excess free PEG was removed by diafiltration using 50 mM PBS. The extent of modification of glutaminase was 74% as determined by trinitrobenzenesulfonate titration of amino groups (see Habeeb, 1966 above). The PEG-glutaminase product preserved 81% of the enzymatic activity of the parent glutaminase.

Example 5

Preparation of PEG-Trypsin: A solution of bovine pancreatic

trypsin (Boeringer-Mannheim, 120 mg in 20 ml), that was dialyzed overnight at 4°C against 20 mM $CaCl_2$ in 1 mM HCl, was brought to 25°C and treated with SC-PEG (600 mg) for 30 min. During this time pH 7.8 was maintained in the reaction vessel by automatic titration with 0.5 N NaOH. The solution was acidified to pH 3 and extensively diafiltered to remove excess of free polymer using 20 mM $CaCl_2$ in 1 mM HCl as a replacement fluid. The modified trypsin had approximately half of the free amino groups of the parent enzyme (7 PEG chains per trypsin molecule) as determined by TNBS titration of amino groups (Habeeb 1966). The PEG-trypsin product preserved 96% of enzymatic activity of the parent enzyme towards N^{α}-benzoyl-L-arginine ethyl ester.

Example 6

Preparation of PEG-Arginase: A solution of bovine liver arginase (Sigma, 90 mg) in 0.1 M NaCl (20 ml) was treated with SC-PEG (1.3 g) at 27°C while pH 8.0 was maintained by automatic titration with 0.5 N NaOH. After 30 min. the reaction mixture was diafiltered using 50 mM PBS as a replacement fluid. Approximately 64% of the amino groups of the native arginase were modified (56 PEG chains per arginase molecule). The PEG-arginase product retained 70% of specific activity of the native enzyme when assayed with 2,3-butanedione (BUN-urea reagent) at pH 9.5.

Example 7

Other polypeptides, including chymotrypsin, asparaginase, and adenosine deaminase, have been modified with SC-PEG using the procedures set forth herein.

The methods of using SC- and/or BSC-functionalized polyalkylene oxides, such as PEG and its copolymers are

generally applicable to the preparation of other modified polypeptides and other biologically active components having amino groups.

While the present invention has been described by reference to N-hydroxysuccinimide derivatives, it will be obvious to those skilled in the art that other N-hydroxydicarboximides may be substituted therefor. Typical of such derivatives are N-hydroxyphthalimide, N-hydroxyglutarimide, N-hydroxytetrahydrophthalimide, N-hydroxy-5-norbornene-2,3-dicarboximide or other N-acyl derivatives of hydroxylamine.

As seen in Scheme 2, the product of protein modification using SC-PEG has PEG-chains grafted onto the polypeptide backbone through carbamate (urethane) linkages. Greater stability of the urethane linkage relative to the ester bond produced upon use of SS-PEG (see Scheme 1) was expected to prove a key difference between the two activated PEG's and the corresponding PEG-protein conjugates. Our studies indeed confirmed this expectation. Figure 1 shows the results of GF-HPLC measurements of the amounts of free mPEG produced as a result of incubation of PEG-BSA conjugates derived from each of the activated PEG's. Considerably higher stability of the SC-PEG-derived conjugate is apparent.

To estimate the reactivities of SC-PEG and SS-PEG, we performed kinetic measurements of hydrolysis of the activated polymers in phosphate buffer and their aminolysis by Nα-acetyl-lysine (NAL). The results of these experiments are summarized in Table 1. It is clear from these data that SS-PEG is a more reactive reagent than SC-PEG. The difference in hydrolysis rates was larger than the difference in aminolysis rates; consequently, SC-PEG showed more favorable K_{am}/K_h ratios. The slower hydrolysis of SC-PEG

was also manifested in superior storage stability of the reagent (Figure 2).

Reactivity of the activated PEG's as a function of pH was also determined using NAL as a model for the ϵ-amino group of a protein. We reacted each activated PEG with an equimolar amount of NAL at different pH's, and measured the unreacted NAL using the TNBS-assay (Figure 3). The optimal pH for use of SC-PEG was found to be about 9.3. It is not advisable to use SS-PEG at pH>8.0, due to the limited stability of PEG-succinate ester. However, even at pH values less than 8.0 this activated PEG was found to be very reactive.

Both reagents showed high reactivity towards Trypsin yielding comparably modified enzyme derivatives in mild conditions (pH 7.5 - 8.5) within 30 min. The products were purified by diafiltration, and the degrees of modification were determined by fluorometric assay, according to Stocks, et al. [(1986) Anal. Biochem. 154, 232].

All PEG-modified trypsin derivatives were essentially lacking (<1% of native) proteolytic activity as determined by the Azocoll assay [Chavira, et al. (1984) Anal. Biochem. 136, 446]. The specific activities of representative SS-and SC-PEG modified trypsins towards low molecular weight substrates are summarized in Table 2. The modifications produced hardly any changes in esterolytic activities towards benzoyl-L-arginine ethyl ester, but did enhance the activities towards p-nitroanilides. Michaelis-Menten kinetic constants for several SC- and SS-PEG modified trypsins were measured using ZAPA as the substrate. These results, summarized in Figure 6, indicate that, while V_{max}, K_{cat} and K_{cat}/K_m were increasing gradually with the extent of modificaitons, K_m values were decreasing.

Enhancement in tryptic activity towards simple substrates sometimes is attributed to acylation of tyrosyl residues on the emzymes [Trenholm, et al. (1969) Biochemistry, 8, 1741; Nakata, et al. (1972) J. Biochem. 72,281]. The PEG-trypsin derivatives were checked for any presence of acylated tyrosyl residues. Treatment of SC-PEG modified trypsin derivatives with hydroxylamine did not produce any change in UV spectra, indicating that SC-PEG did not acylate tyrosyl residues of the enzyme. However, when SS-PEG modified trypsin derivatives were subjected to NH_2OH treatment, UV absorbance increments (DA_{278}) were consistently observed. These spectral changes were initially interpreted as evidence for the presence of acylated tyrosines. Upon closer examination of these spectral changes, it was determined that NH_2OH-induced absorbance increments were centered around 260 nm and not around 275-280 nm (the characteristic for deacylated tyrosyl residues). In a different set of experiments, presence of N^{α}-acetyl-tyrosine (NAT) in the reaction between NAL and each one of the activated PEG's showed little influence on the amount of amine reacted (Table 3). On the other hand, the results presented in Table 3 show that SC-PEG is slightly more selective towards amine than SS-PEG.

As compared to SS-PEG, SC-PEG is a less reactive yet more selective reagent. This is evidenced by its higher K_{am}/K_h ratios, better storage stability and lower interference of NAT in SC-PEG reactions with amine. SC-PEG is a sufficiently reactive reagent to produce PEG-protein conjugates under mild conditions within 30 min. SC-PEG can be used in a broader pH range than SS-PEG, showing the highest reactivity at pH=9.3. PEG-protein conjugates obtaining through use of SC-PEG are chemically more stable than SS-PEG derived conjugates. The PEG-Trypsin conjugates produced by both activated PEG's have very similar

properties: They show no proteolytic activity, well preserved esterolytic activity, and dramatically increased activity towards p-nitroanilide substrates. Michaelis-Menten constants of the modified enzymes indicate that the attachment of PEG to trypsin causes an increase in both the rate of turnover of ZAPA and its affinity towards the modified enzymes. These increases in amidolytic activity appear to be unrelated to acylation of tyrosyl residues.

Table 1

Comparison of first order rate constants for hydrolysis (K_h) and aminolysis (K_{am}) of SC-PEG and SS-PEG[a]

pH	Temp. (°C)	Hydrolysis: K_h [b] (min^{-1}) x 10^3 and [$t_{1/2}$ (min)] SC-PEG	Hydrolysis: K_h [b] (min^{-1}) x 10^3 and [$t_{1/2}$ (min)] SS-PEG	Aminolysis: K_{am} [c] (min^{-1}) x 10^3 and [K_{am}/K_h] SC-PEG	Aminolysis: K_{am} [c] (min^{-1}) x 10^3 and [K_{am}/K_h] SS-PEG
7.0	4	0.87 [793]	1.84 [376]	2.64 [3.0]	3.74 [2.0]
	27	6.05 [115]	10.4 [67]	26.4 [4.4]	41.4 [4.0]
	37	14.2 [49]	25.9 [27]	81.7 [5.8]	104 [4.0]
7.4	22	5.37 [129]	10.7 [65]	29.1 [5.4]	42.7 [4.0]
	27	9.0 [77]	16.0 [43]	48.6 [5.4]	73.6 [4.6]
	37	19.3 [36]	37.6 [18]	145 [7.5]	193 [5.1]
7.8	4	1.37 [505]	2.58 [268]	12.4 [9.1]	15.0 [5.8]
	27	10.3 [67]	21.6 [32]	130 [12.6]	152 [7.0]
	37	21.8 [32]	48.8 [14]	226 [10.6]	267 [5.5]

[a] All the measurements were performed by following the appearance of N-hydroxysuccinimide anion (-OSu) at 260 nm in 0.8 M sodium phosphate; concentration of PEG-bound succinimidyl active acyl at time zero $[SX\text{-}PEG]_o$ was 0.1 mM; in aminolysis experiments concentration of N^{α}-acetyl-lysine at time zero $[NAL]_o$ was 3 mM.

[b] $K_h = Rate_h/[SX\text{-}PEG]_o$, where $Rate_h = dA_{260}/dt \times 1\ E_{260} \times 1F$; $E_{260} = 8500\ M^{-1}\ cm^{-1}$ is an extintion coeficient of -OSu; and $F = [-OSu]/([HOSu] + [-OSu)] = (1 + 10^{6.0-pH})^{-1}$.

[c] K_{am} = Total Rate/$[SX\text{-}PEG]_o$ - K_h. The Total Rate in aminolysis experiments was calculated the same way as $Rate_h$ in hydrolysis experiments.

Table 2

SUMMARY OF MODIFICAITON, ESTEROLYTIC ACTIVITY, AND AMIDOLYTIC ACTIVITY DATA FOR TYPSIN AND ITS mPEG DERIVATIVES

Trypsin Derivatives[a]	Modif[b] (%)	BAEE[c] (u/mg)	% Native	BAPA[d] (u/mg)	% Native	ZAPA[d] (u/mg)	% Native
Native Trypsin	0	92.4	100	1.26	100	7.81	100
SC-PEG^N-Trypsin							
N = 6	42.3	103	112	2.26	179	15.3	196
N = 7	45.8	87.9	95.1	2.38	188	17.5	224
N = 9	58.8	90.1	97.5	2.67	212	18.9	242
N = 12	77.9	85.1	92.2	3.83	304	25.5	326
SS-PEG$_h$-Trypsin							
N = 7	44.8	102	110	3.25	258	18.8	241
N = 12	770	94.3	102	4.34	344	24.7	316

[a] For SX-PEG$_h$-Typsin, N = 15 x (% Modif)/100 and is rounded to the nearest integer.

[b] The percent of amino groups modified was detrmined by the fluorescamine assay [Stocks, et al. (1986) Anal. Biochem. 154, 232]

Table 2 (continued)

[c] The BAEE (Nα-benzoyl-L-arginine ethyl ester) trypsin assay was done at pH 7.8, 37°C w/ a substrate conc'n of 0.5 mM. The extinction coefficient was $\epsilon_{283} = 808\ M^{-1}\ cm^{-1}$ [Kezdy, et al. (1965) Biochemistry 4, 99].

[d] The BAPA (Nα-benzoyl-D, L-arginine-p-nitroanilide) and ZAPA (Nα-CBZ-L-arginine-p-nitroanilide) amidolytic assays were done w/ a substrate conc'n of 1 mM in 50 mM Tris-HCl pH 8.1, 10 mM $CaCl_2$, at 37°C. The extinction coefficient for p-nitroaniline, $\epsilon_{410} = 8800\ M^{-1}cm^{-1}$, was used in both assays.

Table 3

MICHAELIS-MENTEN CONSTANTS FOR THE AMIDOLYTIC ACTIVITY OF NATIVE TRYPSIN AND ITS mPEG DERIVATIVES[a]

Trypsin Derivatives	Km (mM)	V_{max} (μM/min)	K_{cat} (min^{-1})	K^{cat}/K^{m} (mM^{-1} min^{-1})
Native Trypsin	1.08	15.7	378	349
SC-PEG_h-Trypsin				
N = 7	0.29	19.6	470	1626
N = 9	0.21	20.2	484	2290
N = 12	0.11	22.9	549	4973
SS-PEG_h-Trypsin				
N = 7	0.21	18.6	447	2172
N = 12	0.13	22.5	539	4159

[a] The measurements took place at 37°C with a constant trypsin protein concentration of 1.0 μg/ml (via Bluret assay). Nα-carbobenzoxy-L-arginine-p-nitroanilide (AZPA) was used as a substrate in concentrations varying from 0.02 to 1.71 mM in 50 mM Tris-HCl pH 7.8, 10 mM calcium chloride. The constants were calculated from Lineweaver-Burk plots of the initial rates of the appearance of p-nitroaniline (ϵ_{410} = 8800 M^{-1} cm^{-1}).

Table 4

EFFECT OF Nα-Ac-L-Tyr (NAT) ON THE EXTENT OF REACTION OF SS-PEG AND SC-PEG WITH Nα-Ac-L-Lys (NAL)

	% of NAL Reacted with[a]	
NAT/NAL	SS-PEG	SC-PEG
0	78.55 (100)[b]	53.75 (100)
1.0	77.15 (98.2)	55.25 (103)
2.5	74.50 (94.8)	52.55 (97.8)
5.0	68.50 (87.2)	48.60 (90.4)

[a] To triethanolamine-borate buffer (0.3 M, pH 8.1) the following was added: a solution of NAL (50 mM, 0.1 ml) and a solution of NAT (100 mM, volume corresponding to the ratios given in the table and bringing the combined volume to 1.1 ml) both in the same buffer, and lastly the appropriate activated PEG in CH_3 CN (50 mM active acyl, 0.1 ml). The resultant solution was vortexed and incubated at 28°C for 1 hr. A mixture of the same components but leaving out SX-PEG was used as a control. The TNBS assay version of Snyder, et al. [(1975) Anal. Biochem. 64, 284] was used to determine the unreacted NAL.

[b] The numbers given in the parentheses represent the values of percent of NAL reaction divided by the percent of NAL reaction when NAT/NAL = 0.

What is claimed is:

1. A compound having the structure

$$R_1-(O-R_2)_a-(O-R_3)_b-(O-R_4)_c-O-\overset{O}{\overset{\|}{C}}-O-R_5$$

wherein R_1 is H-, H_3C-, an N-dicarboximide group, or any other functional group;

wherein each R_2, R_3, and R_4 is an alkyl group which may be straight, branched, disubstituted, or unsubstituted, and wherein each R_2, R_3, and R_4 may be independently the same as, or different from, the others of R_2, R_3, and R_4;

wherein R_5 is an N-dicarboximide group; and

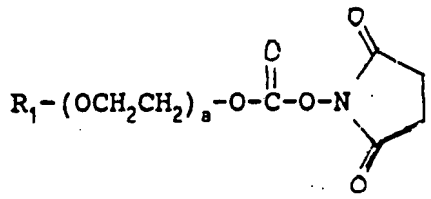
wherein a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b, and c is between 10 and 1000.

2. A compound of claim 1, wherein each of R_2, R_3, R_4 is CH_2CH_2, CH_2CHCH_3, or $CH_2CH_2CH_2CH_2$.

3. A compound according to claim 1 wherein each of R_1 and R_5 is an N-dicarboximide group selected from the group consisting of N-succinimide, N-phthalimide, N-glutarimide, N-tetrahydrophthalimide and N-norbornene-2,3-dicarboximide and where R_1 and R_5 may be the same or different.

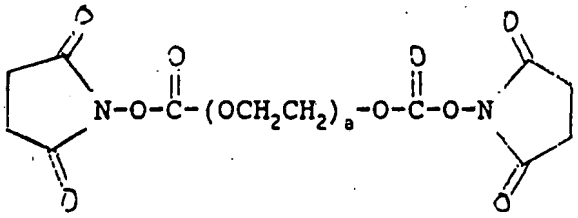
4. A compound according to claim 3 wherein R_5 is an N-succinimide group.

5. A compound of claim 1 having the structure



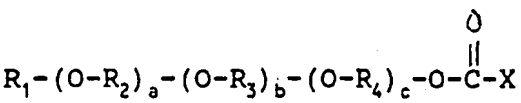
where R_1 is H_3C-, H-, an N-dicarboximide group, or any other functional group; and a is an integer between 10 and 1000.

6. A compound of claim 1 having the structure



wherein a is an integer between 10 and 1000.

7. A process for preparing a compound of claim 1, which comprises reacting a compound having the structure



wherein R_1 is H-, H_3C-, N-dicarboximide group, or any other functional group;

wherein each R_2, R_3, and R_4 is an alkyl group which may be straight, branched, disubstituted, or unsubstituted, and wherein each R_2, R_3, and R_4 may be independently the same as, or different from, the others of R_2, R_3, and R_4;

wherein X is a halogen;

wherein a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b, and c is between 10 and 1000.

with an N-hydroxydicarboximide in the presence of a base.

8. A process of claim 7, wherein the N-hydroxydicarboximide is N-hydroxysuccinimide.

9. A process according to claim 7 wherein the halogen is chlorine and the base is triethylamine.

10. A modified polypeptide comprising a polypeptide having bound thereto at least one polymer having the structure

$$R_1-(O-R_2)_a-(O-R_3)_b-(O-R_4)_c-O-\overset{O}{\overset{\|}{C}}-$$

wherein R_1 is H-, H_3C-, or an N-dicarboximide group, or any other functional group;

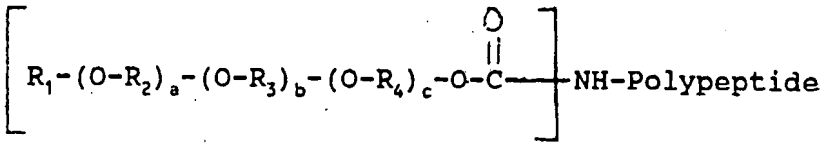
wherein each R_2, R_3, and R_4 is an alkyl group which may be straight, branched, substituted, or unsubstituted, and wherein each R_2, R_3, and R_4 may be independently the

same as, or different from, the others of R_2, R_3, and R_4;

wherein a is an integer between 1 and 1000 and each of b and c is an integer between 0 and 1000, and the sum of a, b, and c is between 10 and 1000.

wherein each polymer is covalently bound to an amine group of the polypeptide by a urethane linkage.

11. A modified polypeptide of claim 11, having the structure



12. A modified polypeptide of claim 12, wherein the polypeptide is bovine serum albumin, glutaminase, trypsin, arginase, chymotrypsin, asparaginase, or adenosine deaminase.

13. A process for preparing a modified polypeptide comprising reacting a polypeptide with the compound of claim 1 at a pH in the range of about 5.8 - 11.

14. A process of claim 12, wherein the pH is about 9.3.

Figure 1

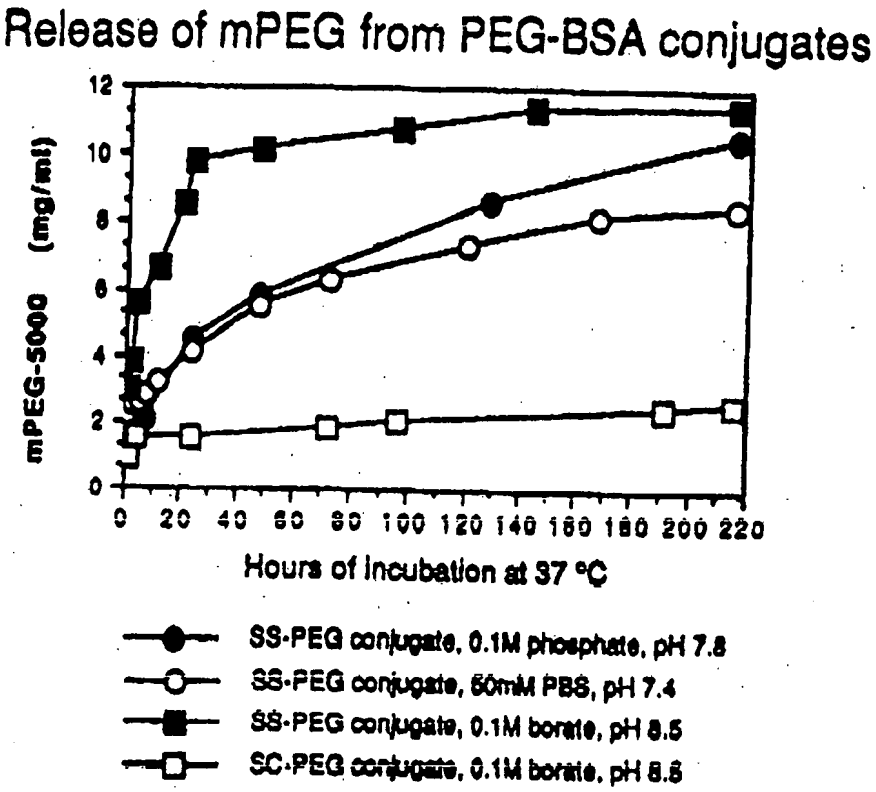

Release of mPEG from PEG-BSA conjugates
mPEG-5000 (mg/ml)
0
2
4
6
8
10
12
0 20 40 60 80 100 120 140 160 180 200 220
Hours of Incubation at 37 °C
SS-PEG conjugate, 0.1M phosphate, pH 7.8
SS-PEG conjugate, 50mM PBS, pH 7.4
SS-PEG conjugate, 0.1M borate, pH 8.5
SC-PEG conjugate, 0.1M borate, pH 8.5

Figure 2

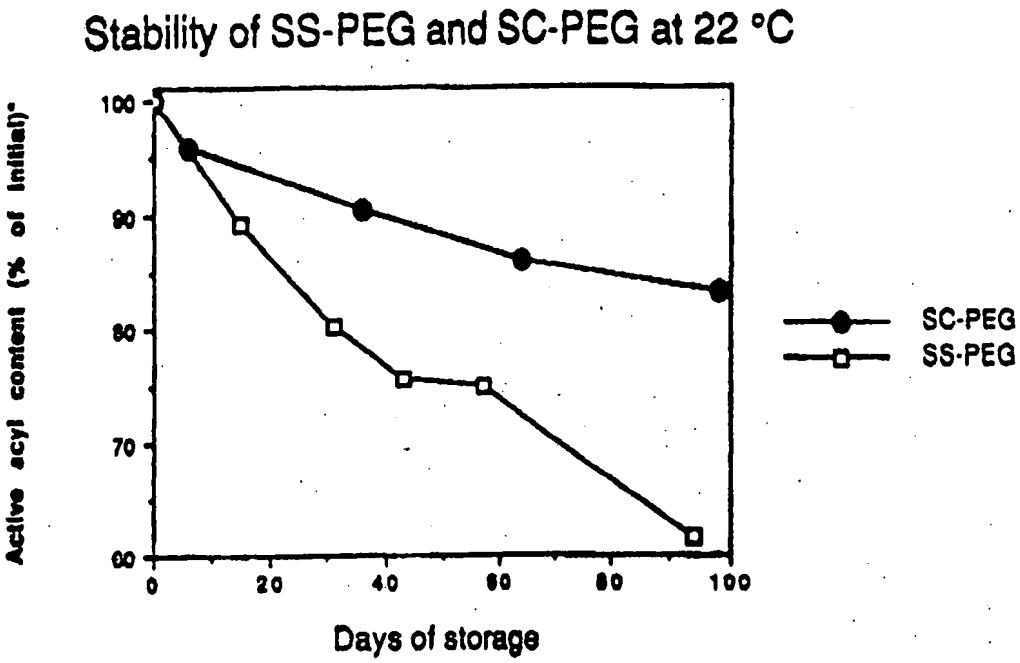
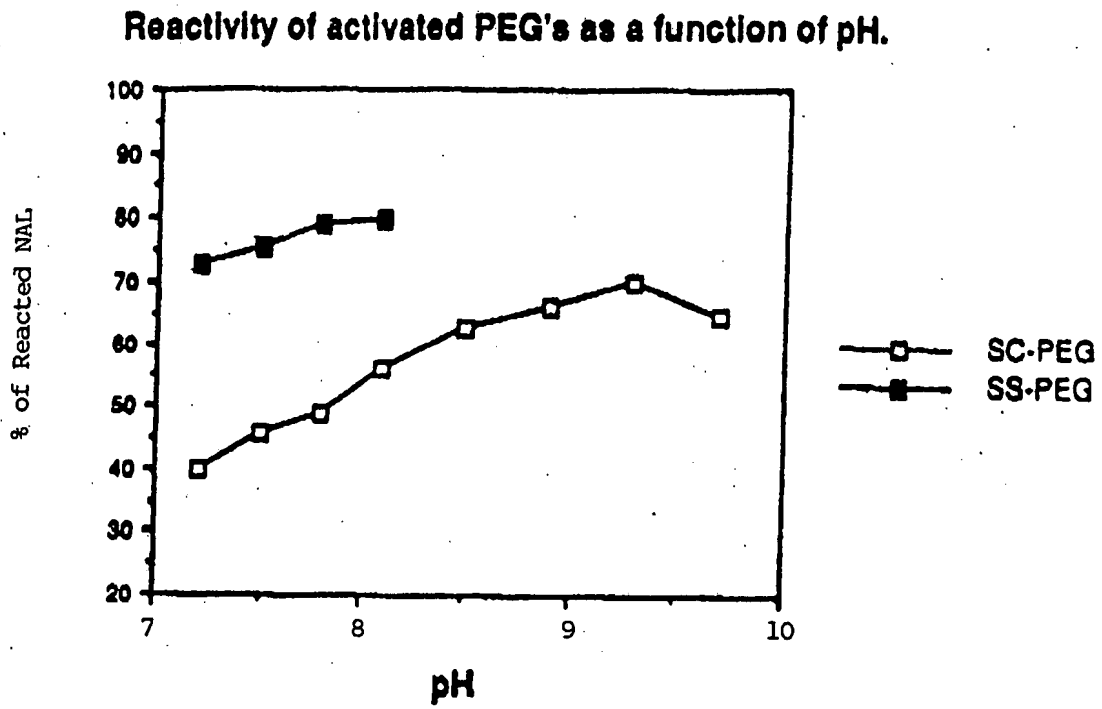

Stability of SS-PEG and SC-PEG at 22 °C
Active acyl content (% of initial)*
100
90
80
70
60
0
20
40
60
80
100
Days of storage
SC-PEG
SS-PEG

Figure 3


Reactivity of activated PEG's as a function of pH.
% of Reacted NAL
100
90
80
70
60
50
40
30
20
7
8
9
10
pH
SC-PEG
SS-PEG

INTERNATIONAL SEARCH REPORT

International Application No PCT/US90/02133

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [3]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): C07D 207/46, 403/12
US Cl.: 548/547, 520

II. FIELDS SEARCHED

Minimum Documentation Searched [4]

Classification System	Classification Symbols
U.S.	548/520,547; 435/181,174,188,177

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [5]

APS AND CA COMPUTER SEARCH FILES

III. DOCUMENTS CONSIDERED TO BE RELEVANT [14]

Category *	Citation of Document, [16] with indication, where appropriate, of the relevant passages [17]	Relevant to Claim No. [18]
A	US, A, 4,248,786 (BATZ et al.) 03 February 1981, see abstract and columns 2 and 6.	1-6
A	Cancer Biochem. Biophys., Volume 7, issued 1984, A. Abuchowski et al., "Cancer therapy with chemically modified enzymes. I. antitumor properties of polyethylene glycol-asparaginase conjugates", see page 177.	1-5
A,P	US, A, 4,891,430 (Barcelo et al.) 02 January 1990, see abstract and columns 4, 5 and 7.	1-9

* Special categories of cited documents: [15]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search [2]	Date of Mailing of this International Search Report [2]
31 August 1990	26 SEP 1990
International Searching Authority [1]	Signature of Authorized Officer [20]
ISA/US	Kay K. Kim

Form PCT/ISA/210 (second sheet) (May 1986)

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers . because they relate to subject matter [1] not required to be searched by this Authority, namely:

2. ☐ Claim numbers . , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [1], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

Group I. Claims 1-9 drawn to a compound and process of making thereof classified in Classes 546 and 548 subclasses 188 + and 435 + respectively.

Group II. Claims 10-14 drawn to a modified polypeptide and method of making thereof classified in Classes 435 and 530 subclasses 188 and 410 respectively. See Attach.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

Claims 1-9 wherein R_1= H, CH_3, N-succinimide and R_5= N-succinimide.

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

PCT/US90/02133
ATTACHMENT TO FORM PCT/ISA/210 Part VI
OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING.

Group I encompass distinct n-dicarboximide moity(ies) with regards to both number and types as follow in order of classification priority: 1) glutarimide with any other species of N-dicarboximide as recited in claim 3 or with H, CH3 of claim 1; 2) N-norbornene-2, 3-dicarboximide with H, CH3, N-norbornene-2, 3-dicarboximide, N-phthalimide, N-tetrahydrophthalimide or N-succinimide; 3) N-phthalimide or N-tetrahydrophthalimide with H, CH3, N-phthalimide, N-tetrahydrophthalimide or N-succinimide; 4) N-succinimide with H, CH3, or N-succinimide.

Group II encompass distinct polypeptide as follows: 1) bovine serum albumin and 2) enzymes of claim 13.